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Optimization of a simplified method for fruit phenolic extraction and analysis to be used in olive breeding.

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Phenolic compounds are generally accepted as key components of virgin olive oil with a great impact on its organoleptic and health promoting properties. However, olive phenolic components are not commonly used as quality traits in olive breeding programs mainly due to the difficulties of evaluating a large number of new genotypes with very little oil production at the early stages of breeding. In the present work, we describe and optimize a fast and reliable method for the extraction and further analysis of the main phenolic compounds found in olive fruit. The analytical methodology has been validated with the two most relevant olive varieties grown in Spain, "Picual" and "Arbequina", which possess very different phenolic profiles. The significant correlation found between specific phenolic compounds, or groups of phenolic compounds in the olive fruit analyzed with this method, and the phenolic content of virgin olive oil suggest that this method may be a very useful predictive tool which could prevent the selection of olive genotypes whose oils will never reach an optimum phenolic content.

Keywords: *Olea europaea* L**.,** olive, fruits, virgin olive oil, phenolic compounds, breeding.

INTRODUCTION

Virgin olive oil (VOO) represents the main lipid source in the Mediterranean diet. It is one of the oldest known plant oils and due to its unique extraction process contains significant amounts of biologically active metabolites (e.g., phenolic compounds, tocopherols, sterols, volatile compounds and pigments) which enhance its nutritional and organoleptic properties (Visioli and Bernardini, 2013). In this sense, it is generally accepted that the phenolic compounds are the VOO components most directly associated with its health related properties (Bernardini and Visioli, 2017). In fact, the scientific evidences on the ability of VOO phenolic components to reduce chronic inflammation and oxidative damage has led the European Union to approve a health claim on olive oil polyphenols which may be applied only for oils containing at least 250 mg/ kg of hydroxytyrosol (3,4-DHPEA) and its derivatives (Commission Regulation (EU) 432/2012). Besides their health promoting properties, VOO phenolic components also have important organoleptic implications given that they are associated to the bitter and pungent sensory notes characterizing VOO (Mateos et al., 2004).

VOO profile comprise a wide range of phenolic compounds belonging to different chemical classes such as secoiridoids, lignans, flavonoids and simple phenolic alcohols and acids. There are many differences in the phenolic profiles among olive cultivars (García-Rodríguez et al., 2017; García-González, Tena, and Aparicio, 2010) due to genetic factors

(Pérez et al., 2014), the agronomic conditions (Romero and Motilva, 2010), and/or the industrial procedures used during the oil extraction (El Riachy et al., 2011). The biosynthesis of the phenolic compounds responsible for the functional and sensory quality of VOO takes place when enzymes and substrates are brought into contact during the milling of olive fruits, the first step in the VOO extraction process. Previous studies have demonstrated that the composition and biochemical status of the olive fruit are the most important variables defining the organoleptic and nutritional quality of the oil. Thus, the phenolic profile of VOO mainly depends on the phenolic content of the olive fruit (Gómez-Rico et al., 2008) and the activity of hydrolytic and oxidative enzymes such as β-glucosidase, polyphenol oxidase and peroxidase (Romero-Segura et al., 2011). Secoiridoid compounds are the key phenolic components in all olive products. Oleuropein, ligstroside and demethyloleuropein are the main phenolic glycosides found in the olive fruit, and their hydrolytic derivatives, identified as the dialdehydic forms of decarboxymethyloleuropein and ligstroside aglycones (3,4-DHPEA-EDA and *p*-HPEA-EDA, respectively), and the aldehydic forms of oleuropein and ligstroside aglycones (3,4-DHPEA-EA and *p*-HPEA-EA, respectively), are the most abundant phenolic components in most VOOs.

Due to their health promoting and organoleptic properties, phenolic compounds are currently being used as quality markers for VOO. However, phenolic components are not commonly used as quality traits in olive breeding programs, mainly due to the difficulties for evaluating oil phenolic composition in large number of samples and the limited knowledge on the genetic and environmental factors that may influence phenolic composition. In this sense, recent studies have described significant correlations between the composition of olive fruits and oils for components such as fatty acids, sterols, tocopherols or squalene (De la Rosa et al., 2016). In order to predict the phenolic composition of the oil from the analysis of the fruits, it is important to have a reliable and simple analytical methodology to characterize phenolic composition of olive fruits.

Despite the numerous techniques applied and the large amount of published literature on the subject, olive cultivars are far from being fully characterized regarding phenolic compounds (García-Rodríguez et al., 2017). Traditionally, conventional liquid-liquid or solid phase extraction methods have been used to extract phenolic compounds from olive fruits and oils. Methanol/water is probably the solvent most used in these protocols usually involving combination of several extracts, and further washing, evaporation and dissolution steps but the use of other solvents have also been reported (Del Río et al., 2003; García et al., 2016). In a similar way, different sample processing techniques have been used in these extraction protocols: fresh olive tissue (Romero et al., 2017), frozen tissue (Gómez-Rico et al., 2008) or freeze-dried samples (Klen et al., 2015).

The aim of this study was to describe a fast and reliable method for the extraction and analysis of olive fruit phenolic compounds which could be applied to a large number of samples, such as those usually associated to olive breeding programs, and that olive breeders could use as a predictive tool.

MATERIAL AND METHODS

Plant material

Olive fruits (*Olea europaea* cv. Picual and Arbequina) used for the optimization of the extraction method were cultivated at the experimental fields of Instituto de la Grasa (Seville, Spain) and collected at an average maturity index of 2.5 (turning stage). Correlations studies among different phenolic compounds were carried out with 25 samples of fruits and oils obtained from Picual and Arbequina trials planted in different olive growing areas of Andalusia.

Olive oil extraction

Olive oil was extracted using an Abencor analyzer (Comercial Abengoa, S.A., Seville, Spain) that simulates the industrial process of VOO production on a laboratory scale (Martínez, et al., 1975). Processing parameters have been precisely described in a previous study (Pérez et al., 2014).

Extraction of fruit phenolic compounds

Two types of samples were used for the extraction of phenolic compounds, fresh pulp tissue and pulp tissue frozen with liquid N_2. Frozen samples were stored at -80ºC for up to 3 months. Both samples were process in the same way: longitudinal pieces of mesocarp tissue were cut from 20 olive fruits and grinded in dimethyl sulfoxide DMSO (6 ml/g of fruit) or submerged in the same solvent at 4ºC for different periods of time (24, 48, 72h, 1 week and 1 month). Syringic acid (24 µg/ml DMSO) was used as internal standard. All the phenolic extracts obtained were filtered through a 0.45 µm mesh nylon and kept at -20ºC until HPLC analysis.

Extraction of VOO phenolic compounds

VOO phenolic compounds were isolated by solid phase extraction (SPE) on a diol-bonded phase cartridge (Supelco, Bellefonte, PA) following a previously described procedure (Mateos, et al., 2001).

Analysis of phenolic compounds

Phenolic extracts from fruits and oils were analyzed by HPLC on a Beckman Coulter liquid chromatography system equipped with a System Gold 168 detector, a solvent module 126, an auto sampler module 508 and a Waters column heater module following a previously described methodology (García-Rodríguez et al., 2011). A Superspher RP 18 column (4.6 mm i.d. x 250 mm, particle size 4 µm: Dr Maisch GmbH, Germany) at flow rate 1mL min^{-1} and a temperature of 35 ºC was used for all the analyses. A total of 15 phenolic compounds were analyzed in fruit phenolic extracts: hydroxytyrosol-4-glucoside, hydroxytyrosol-1-glucoside, demethyloleuropein, verbascoside, luteolin-7-glucoside, demethylligstroside, rutin, oleuropein, comselogoside, ligstroside, luteolin 3,4-DHPEA-EA, apigenin and *p*-HPEA-EA. The last four compounds were also analyzed in VOO extracts in which 12 other phenolic compounds were also detected: hydroxytyrosol, tyrosol, vanillic acid, vainillin, p-coumaric acid, hydroxytyrosol acetate, 3,4-DHPEA-DEA, *p*-HPEA-DEA, pinoresinol, acetoxypinoresinol, and ferulic acid. The tentative identification of compounds by their UV-vis spectra was confirmed by HPLC/ESI-qTOF-HRMS. (García-Rodríguez et al., 2017).

Statistical analysis

Data were statistically evaluated using STATISTICA (Statsoft Inc., Tulsa, OK, USA). Correlations among phenols or group of phenols were analyzed using Pearson's correlations. ANOVA and separation of the means was obtained at $p \leq 0.05$ by least significance differences (LSD).

RESULTS AND DISCUSSION

The main goal of this study was to optimize a simple and reliable method to be used for phenolic profiling in a large set of olive samples. DMSO is a dipolar aprotic solvent widely used in pharmacology for its ability to penetrate biological membranes. Due to its activity as free radical scavenger, very useful to avoid oxidation of plant extracts, DMSO has been previously used in the extraction of plant phenolic compounds (Del Río et al., 2003). Table 1 and 2 show the phenolic composition of olive extracts obtained by homogenizing olive pulp

tissue in DMSO or by simple immersion of longitudinal pieces of pulp tissue in DMSO for different periods of time. The content of the main phenolic glycosides analyzed in Picual and Arbequina fruits is consistent with previous data on the phenolic profile of both cultivars (Talhaoui et al, 2016; Romero et al., 2017). Although significantly higher contents of some phenolic compounds were found in the extracts obtained by homogenization of the olive tissue nonsignificant differences were found in the total phenolic content of Arbequina extracts obtained by simple immersion in DMSO for more than 48h (Table1). Despite the significant differences found among Picual extracts (Table 2) it is important to point out that total phenolic content in 48h extracts is only 5% lower than that found in those extracts obtained from homogenized tissue. It is also remarkable that hydroxytyrosol and tyrosol glucosides contents were generally lower in those extracts. Data presented in Table 1 and 2 suggest that simple immersion of pulp tissue in DMSO for more that 48h-72h could be a reliable and fast method for the extraction of olive phenolic compounds. The simplicity of the method, with very little manipulation of the samples and very stable extracts could be a very useful tool to analyze large number of samples. In fact, in order to process a high number of samples, many experimental protocols for phenolic analysis freeze tissue samples with liquid nitrogen and kept them at -80ºC until extraction and analysis. In this sense, it is important to emphasize that freezing of the olive pulp and the subsequent thawing of the tissue causes a significant alteration of the phenolic composition. In a recent study (García-Vico et al. 2017), we have described that olive freezing produces a clear reduction in the phenolic content, with a very important decrease of oleuropein and demethyloleuropein levels and a minor reduction in the content of verbascoside and other olive phenolic glycosides. Although, it is clear that hydrolytic and oxidative reactions could contribute to this phenolic degradation, the alteration induced by freezing seems to be more related to the release of β-glucosidase proteins due to intracellular damage. This enzyme, with very high substrate and product specificity, exhibits maximal specificity towards oleuropein, followed by demethyloleuropein, but is not active on verbascoside (Romero-Segura et al., 2011). Figure 1 shows the differences found in the phenolic profiles of fresh and frozen Arbequina and Picual fruits extracted by immersion in DMSO (72h). Oleuropein, the main phenolic glucoside, reduces its content in frozen olive tissues around 60% in both olive cultivars. The degradation of oleuropein and demethyloleuropein seems to be associated to the freezing process and is independent of the subsequent storage time at -80ºC (data not shown). These data highlight the need of using fresh tissue for fruit phenolic profiling.

In order to evaluate the feasibility of the optimized methodology to be used as a predictive tool in olive breeding 25 samples of olives fruits and their corresponding oils were analyzed. Samples were harvested at different ripening stages from different Picual and Arbequina orchards in Southern Spain. Oils were obtained using the Abencor system under standard conditions, and VOO phenolic compounds extracted and analyzed as described in Material and Methods Section. Pearson‘s correlation coefficients were computed using the data obtained from all the fruits and oils analyzed. Table 3 shows the correlation coefficients found among total phenolic content, total secoiridoid compounds and the main classes of phenolic components found in fruits and oils: oleuropein derivatives (oleuropein and demethyloleuropein), ligstroside derivatives (ligstroside and demthylligstroside), hydroxytyrosol derivatives (3,4-DHPEA-EA, 3,4-DHPEA-EDA, 3,4-DHPEA-Acetate), tyrosol derivatives (p-HPEA-EA, p-HPEA-EDA) and lignans (acetoxypinoresinol and pinoresinol).

Table 3. Pearson correlation coefficients among the main groups of phenolic compounds analyzed in fruits and oils from 25 Picual and Arbequina orchards.

Marked correlations are significant at: * $p < 0.05$

A significant positive correlation was found between total fruit phenolics and total VOO phenolic content (r = 0.758) and even a slightly higher coefficient was calculated for fruit secoiridoids compounds and the total phenolic content of the VOO (r = 0.786). A very high correlation was also found between oleuropein derivatives present in the olive fruit and the hydroxytyrosol derivatives present in the VOO (r = 0.754). On the contrary, a negative correlation was found between ligstroside derivatives in the fruit and tyrosol containing compounds in the oil (r = -0.246).

CONCLUSION

The experimental data reported in this paper suggest that the analytical procedure described here for olive phenolic extraction (immersion of longitudinal fruit tissue pieces in DMSO for 72h at 4ºC) is a simple and reliable procedure for olive phenolic profiling. The excellent correlations found between the phenolic profiles of fruits and oils indicate that this methodology could be used as a predictive tool in olive breeding. Taking into account the well-known influence of the phenolic fraction in the sensorial and health-promoting properties of VOO, the analysis of the fruit phenolic profile can be very useful to identify olive cultivars producing oils with an improved nutritional quality.

		Olive fruit			
		Total phenols	Total Secoiridoids	Oleuropein derivatives	Ligstroside derivatives
Olive oil	Total phenols	* 0.758	*0.786	*0.795	0.361
	Total Secoiridoids	* 0.757	*0.785	* 0.795	0.352
	Hydroxytyrosol der.	* 0.744	*0.746	* 0.754	* 0.532
	Tyrosol der.	0.359	*0.422	* 0.430	-0.246
	Lignans	*0.582	*0.590	*0.585	* 0.388

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Figure captions

Figure 1: Content of the main phenolic glycosides (demethyloleuropein, oleuropein, ligstroside, verbascoside and luteolin-7-glucoside) found in fresh and frozen pulp of Arbequina (A) and Picual (B) olive fruits.

Table 1. Main phenolic compounds analyzed in Arbequina fruits by means of different extraction methods using DMSO.

	Main phenolic compounds in Arbequina fruits (µg/g)							
	Hty-G	Tyr-G	DemO	Oleuropein	Ligstroside	Verbascoside	Luteolin-G	Total
Extraction method								
Grinded tissue	645.7a*	60.1b	7736.0b	21314.1c	1014.0a	3028.1b	1155.4b	31978.5b
24h	704.6b	51.4a	7028.9a	15765.5a	1090.5a	2700.6a	916.4a	28257.9a
48h	739.5c	53.3a	7766.0b	16899.0ab	1162.2b	2920.6b	975.9ab	30516.6b
72h	758.4c	54.4ab	7930.0b	17048.3ab	1197.4b	2940.5b	1039.6ab	30700.1b
1 week	749.3c	54.5ab	7939.2b	16818.5ab	1196.5b	2911.0b	1030.3ab	30699.4b
1 month	817.1d	47.9a	8089.2c	17704.8b	1258.5c	3023.2b	1051.3ab	31992.9b

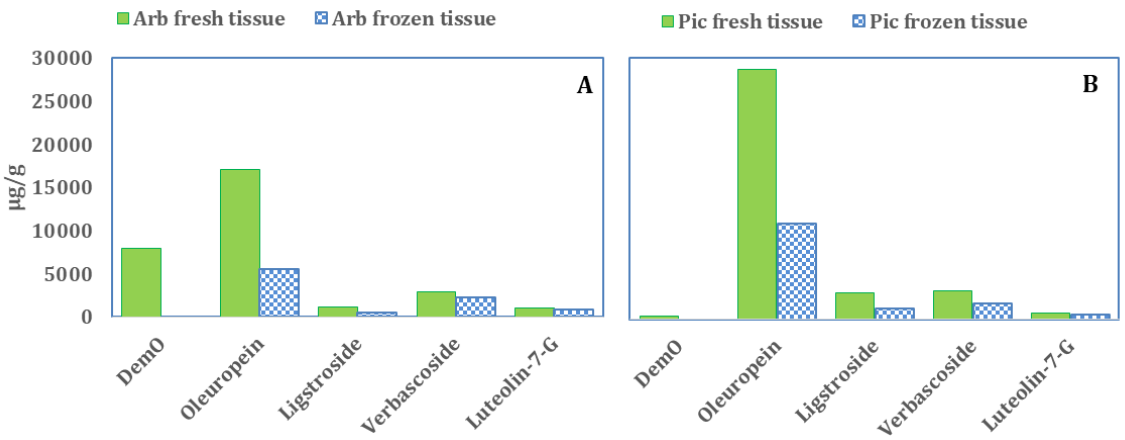
* Values represent the mean of three independent analyses. Different letters in the same column indicate significant differences (p≤0.05)
**Hty-G (hydroxytyrosol-4-glucoside); Tyr-G (Tyrosol-glucoside); DemO (demethyloleuropein); Luteolin-G (luteolin-7-glucoside)

Table 2. Main phenolic compounds analyzed in Picual fruits by means of different extraction methods using DMSO.

	Main phenolic compounds in Picual fruits (µg/g)							
	Hty-G	Tyr-G	DemO	Oleuropein	Ligstroside	Verbascoside	Luteolin-G	Total
Extraction method								
Grinded tissue	353.1a	30,2a	450.5ab	32197,5d	3359,5c	3562.3b	918.7b	40872.3d
24h	492.9b	45.0b	428.9a	29408.7a	3149.9a	3351.8a	739.2a	37616.2a
48h	509.6bc	52.0c	456.5abc	30422.5abc	3232.1b	3429.6ab	768.6a	38871.0abc
72h	512.1c	52.8c	466.8bc	30666.6bc	3248.5b	3488.1ab	794.2ab	39229.2bc
1 week	505.3bc	53.1c	460.3ab	29820.8ab	3208.1ab	3349.1a	767.1a	38163.7ab
1 month	551.0d	57.2d	497.9c	30985.9c	3402.5c	3426.3ab	781.4a	39702.2cd

* Values represent the mean of three independent analyses. Different letters in the same column indicate significant differences (p≤0.05)
**Hty-G (hydroxytyrosol-4-glucoside); Tyr-G (Tyrosol-glucoside); DemO (demethyloleuropein); Luteolin-G (luteolin-7-glucoside)

Figure 1


Arb fresh tissue
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Ligstroside
Verbascoside
Luteolin-7-G